Immunohistochemical localization of enkephalin-like substance in the central nervous system of the amphioxus

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Summary. The localization of enkephalin-like materials in the central nervous system of the amphioxus, *Branchiostoma belcheri*, was studied by immunohistochemical techniques, employing antisera raised in rabbit against synthetic methionine-enkephalin and leucine-enkephalin, respectively. The perikarya and nerve fibers showing methionine-enkephalin-like immunoreactivity were distributed widely in the brain and spinal cord. Furthermore, immunoreactive material was found to accumulate densely in localized spots, around which small tissue spaces were often seen, suggesting possible release of this substance into systemic circulation. These results suggest that this peptide has diverse functions in the amphioxus. No immunoreaction to antiserum against leucine-enkephalin was detected in the central nervous system of the amphioxus.

Introduction

The development of immunohistochemical techniques has made it possible to investigate the phylogenetic distribution of various neuropeptides. In protochordates, immunohistochemical studies on distribution of neuropeptides are rather limited. Immunoreactive luteinizing hormone-releasing hormone and oxytocin have been demonstrated immunohistochemically in the nervous system (Schreibman *et al.*, 1986 ; Vallet and Ody, 1985) and, by radioimmunoassay, thyrotropin-releasing hormone-like molecule (Jackson and Reichlin, 1974) and calcitonin-like molecule (Girgis *et al.*, 1980) were also found in extracts of the head region of the amphioxus. In the present study, immunohistochemical localization of methionine-enkephalin (Met-enk) and leucine-enkephalin (Leu-enk) was investigated in the central nervous system (CNS) of the amphioxus.

Material and Methods

Young and adult specimens of the amphioxus, *Branchiostoma belcheri*, 1.5 to 6 cm in body length, were used. The animals were collected from the subtidal sand bottom by dredging near the Amakusa Marine Biological Laboratory, Kyusyu University, during April and October. They were fixed for 24 h in Bouin's fluid, Bouin's fluid without acetic acid, 4 % parafolmaldehyde in 0.1 M phosphate buffer or a solution consisting of picric acid, glutaraldehyde and acetic acid (25 : 5 : 1). The tissue was embedded in paraffin and cut

into sagittal, horizontal or transverse sections of 8 μm in thickness.

Immunohistochemistry was performed according to the peroxidase-anti-peroxidase (PAP) method of Sternberger *et al.* (1970) or the double PAP method (Vacca *et al.*, 1975). Deparaffinized sections were incubated in the following solutions : 1) 0.3 % H_2O_2 for 15 min ; 2) normal goat serum (1 : 20) for 30 min; 3) diluted primary antiserum for 20 to 24 h at 4°C ; 4) goat anti-rabbit IgG serum (GAR) (1 : 100) for 30 to 80 min; 5) PAP complex (1 : 100) for 30 to 80 min; and 6) 0.015 % 3,3-diaminobenzidine in Tris buffer (pH 7.6) containing 0.005 % H_2O_2 for 5 to 12 min. In the double PAP method, step 5 was followed by incubation in GAR and PAP complex for 30 min each. Incubation was carried out at room temperature unless otherwise specified. After each incubation, the sections were rinsed in 0.01 M phosphate-buffered saline (pH 7.3) three times for 5 min each. In some cases, sections were counterstained by hematoxylin or fast green.

Working dilutions of the anti-Met-enk antiserum were 1 : 1000 and 1 : 1500 for antisera obtained from UCB-Bioproducts, S. A. (Belgium) and Immuno Nuclear Corp (MN, U. S. A.), respectively. The specificity of immunostaining for Met-enk was tested as follows : 1) preincubation of antiserum with synthetic Met-enk (20 μg/ml antiserum at the working dilution); 2) further dilution (1 : 3) of antiserum of appropriate concentrations. If the intensity of immunostaining clearly decreased in the absorption test and disappeared with dilution of antiserum, immunoreactivity was supposed to be specific to the extent that the parameters were tested. The absorption and dilution tests for the anti-Leu-enk antiserum (UCB-Bioproducts S. A.) were not performed, since no positive immunoreaction was obtained at any dilution (1 : 800, 1 : 1600, 1 : 3200, 1 : 8000).

Results and Discussion

Met-enk-like immunoreactivity

1. Immunoreactive perikarya

Immunoreactive bipolar cells were located on either side of the central canal, along the dorsal portion of the spinal cord (Fig. 1). These cells were distributed throughout the whole length of the spinal cord and their fibers seemed to direct antero- or postero-ventrally. These cells may correspond to RBi cells (Rezius bipolar cells-1) designated by Bone (1960). Slightly below these cells, larger immunostained cells, each possessing a large vacuole, were occasionally encountered. They may correspond to the RBii cells of Bone (1960). Bone assumed that RBii cells represent the young stage of the Rezius bipolar cell (RB cell). Supporting this assumption, all RBi cells were replaced by large immunostained cells, each with a large vacuole, in the young specimens (Fig. 2).

Ventrally to the RB cells, giant multipolar cells, showing strong Met-enk immunoreactivity, lay transversely across the central canal (Figs. 3 and 5). These cells were observed throughout the spinal cord except in the mid region of the body. These cells may correspond to the Rohde cells, whose organization and distribution were described by Rohde in 1890 (cited from Bone, 1960). Bone (1960) surmised that the Rohde cells were both internuncial elements, intervening in the somatic-sensori-motor arc and co-

ordinating elements, controlling the swimming movement of the animal. It is interesting to note that interneurons showing Met-enk immunoreactivity have been widely observed within the CNS of the rat (Finley *et al.*, 1981).

In the area where Rohde cells are distributed, two types of small immunostained cells were occasionally observed. The larger type was often triangular in form, possessing a large vacuole and scarce cytoplasm, which was stained strongly (Fig. 4). Cells of the other type were elongated in form and their perikarya or fibers were often lying transversely across the central canal (Figs. 5 and 6).

2. Immunoreactive fibers

Numerous immunoreactvie fine fibers were observed to run longitudinally, widely throughout the spinal cord (Figs. 3, 5, 6, 7, 8 and 9), although the origin of these fibers could not be determined. Within the white matter, positive fibers were divided into two main groups : dorsal and ventral groups (Fig. 7). The fibers of the former were larger in number than those of the latter. The fibers of dorsal group were distributed rather widely to form the subepithelial bundle (Figs. 7 and 11) or to draw near the dorsal root nerve (Figs. 7 and 8). However, only a few immunopositive fibers seemed to run into the dorsal roots (Figs. 8 and 9). Immunopositive material was often densely accumulated in the limited area of the subepithelial region, slightly ventro-posterior to the entry of the dorsal root. Small tissue spaces were present around the accumulation of the immunopositive material (Fig. 10). This may suggest the neuroendocrine nature of Met-enk-like substance in the amphioxus. In connection with this point, we may note that enkephalins are candidates of neurohormones in mammals : immunoreactive Met-enk is present in the rat median eminence and neurohypophysis (Sar *et al.*, 1978; Finley *et al.*, 1981), and enkephalins play a significant role in the modulation of the secretion of several pituitary hormones (Lien *et al.*, 1976; Dupont *et al.*, 1977; May *et al.*, 1979).

Bone (1960) noted that many branches from the dendrites of a single Rohde cell ran toward the dorsal root. The immunoreactive fibers near the entry of the dorsal root may originate, at least partly, in the Rohde cells, although we could not pursue their dendrites.

The fibers of the ventral group were distributed in the area from the ventro-lateral region of the gray matter to the ventral root (Fig. 7). A small amount of immunostained material was often found to accumulate at the central motor end plate (Figs. 7 and 11). Small tissue spaces were often recognized in contact with such an accumulation. This finding suggests that Met-enk-like substance has some function in the somatic-motor system as a neuromodulator and/or plays some role as a neurohormone in the amphioxus.

A widespread distribution of Met-enk immunoreactivity in the various types of neuronal elements suggests diverse functions of Met-enk-like substance in the amphioxus, as various functions of enkephalins have been suggested in mammals (Finley *et al.*, 1981). In the present study, however, immunoreactive Leu-enk was not detected in the CNS of the amphioxus.

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Explanation of Figures

Fig. 1. RBi cells (arrows), showing a Met-enk-immunoreaction in the dorsal region of the spinal cord. Horizontal section. ×660. **Bar :** 10 μm.

Fig. 2. Met-enk-immunostained RBii cells (arrows), each with a large vacuole, in the dorsal region of the spinal cord of a young specimen. Horizontal section. ×640. **Bar :** 10 μm.

Fig. 3. A Rohde cell (arrowhead) showing a Met-enk-immunoreaction. Immunopositive axons (arrows) are observed in the white matter (W). G, gray matter. Horizontal section. ×640. **Bar :** 10 μm.

Fig. 4. Triangular cell (arrowhead), showing strong Met-enk immunoreactivity. Horizontal section. ×640. **Bar :** 10 μm.

Fig. 5. Small elongated cells (arrows), a Rohde cell (arrowhead) and fiber bundles (double arrows) showing a Met-enk-immunoreaction. Horizontal section. ×640. **Bar :** 10 μm.

Fig. 6. Met-enk-immunostained small cell (arrow) lying transversely across the central canal (*). Double arrow, immunopositive fibers. Horizontal section. ×640. **Bar :** 10 μm.

Fig. 7. Diagramatic transverse section of spinal cord. Met-enk-immunoreactive fibers in the white matter are shown by small dots. d, dorsal group ; v, ventral group ; DR, dorsal root ; EP, central motor end plate ; GF, central giant fiber ; SB, subepithelial bundle ; VR, ventral root. Asterisk, central canal. The area shown by large dots is the gray matter.

Fig. 8. A Met-enk-immunoreactive fiber bundle bifercates into two branches : one proceeds into the dorsal root (double arrow) and another posteriorly (arrows). DR, dorsal root. Left, anterior. Sagittal section. ×660. **Bar :** 10 μm.

Fig. 9. Numerous Met-enk-immunoreactive fibers (the dorsal group) are seen in the white matter (arrows). Positive fibers (double arrow) are present in the dorsal root (DR). Horizontal section. ×660. **Bar :** 10 μm.

Fig. 10. Accumulation of Met-enk-immunoreactive material (arrowhead) near the entry of dorsal root. Small tissue spaces (arrows) are seen around the accumulation. Upper, anterior. Sagittal section. ×660. **Bar :** 10 μm.

Fig. 11. The subepithelial bundle of the positive axons (arrowhead) and accumulation of Met-enk-immunoreactive material near the central motor end plate (EP). Arrow, small tissue space. Transverse section. ×660. **Bar :** 10 μm.


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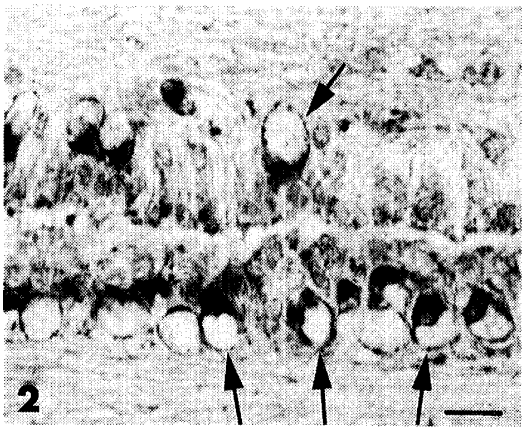
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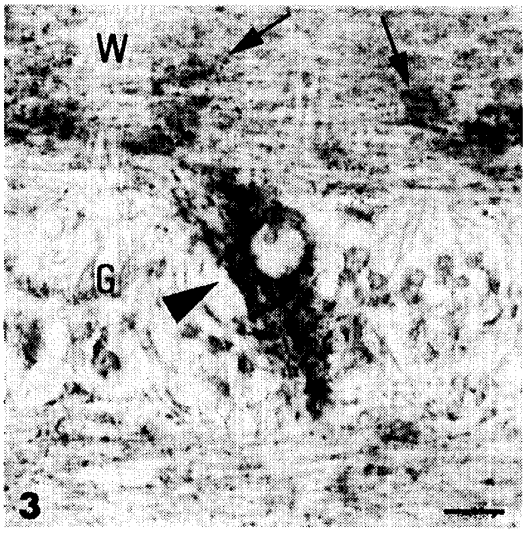
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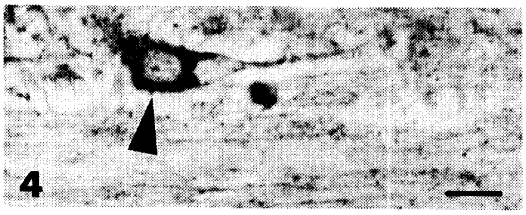
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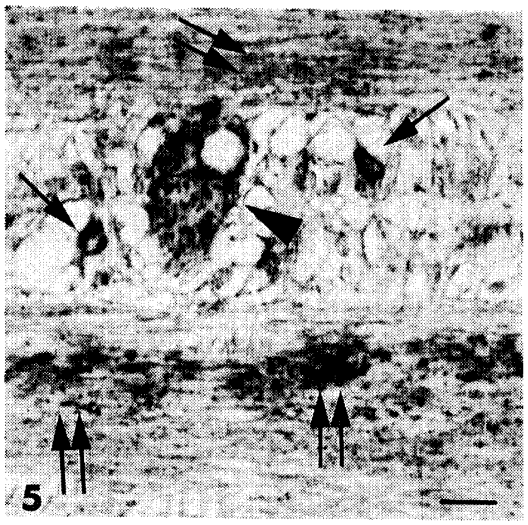
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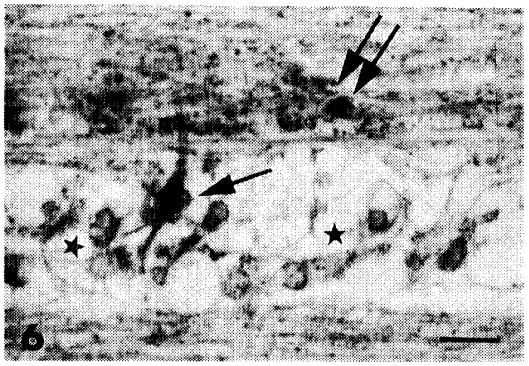
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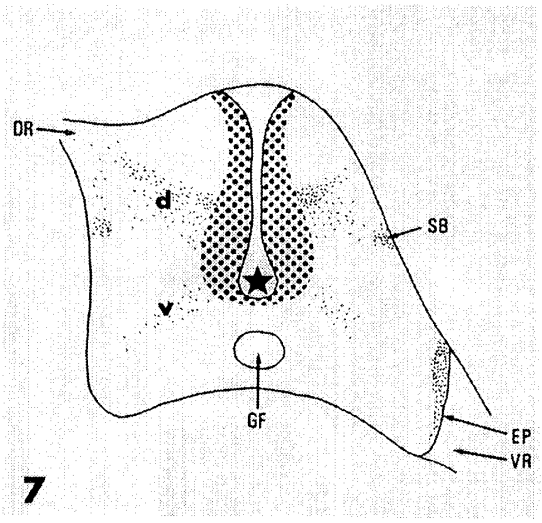
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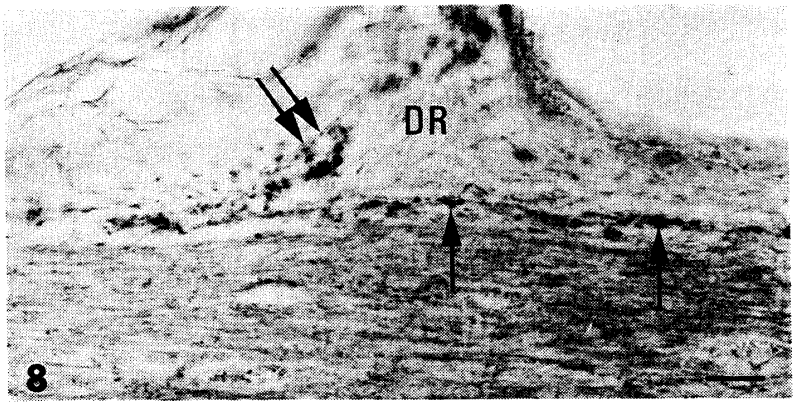
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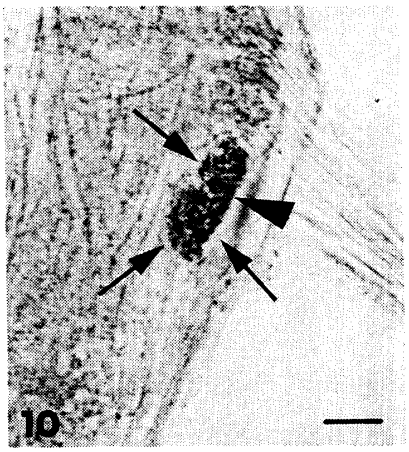
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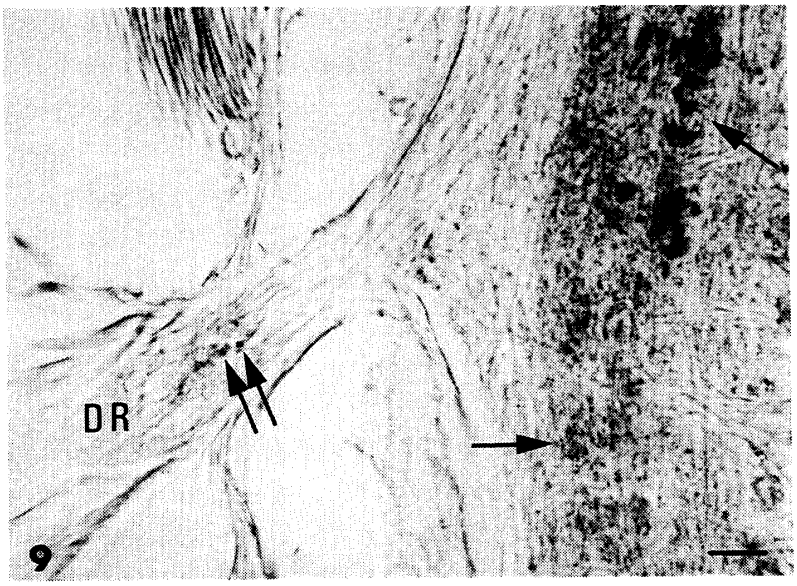
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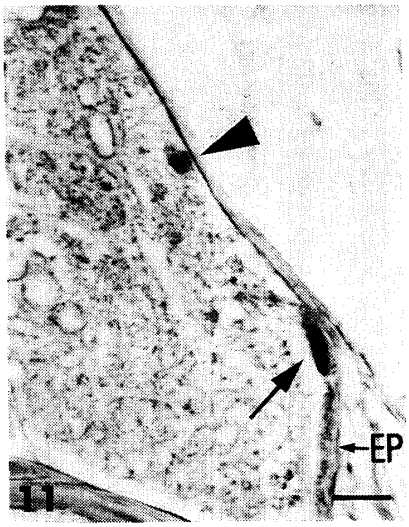
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